AD_____

Award Number: DAMD17-01-1-0185

TITLE: Coactivators and Corepressors in Breast Development and Receptor-Dependent Tumorigenesis

PRINCIPAL INVESTIGATOR: Soo-Kyung Lee, Ph.D.
Ivan Garcia Bassets, Ph.D.

CONTRACTING ORGANIZATION: The University of California, San Diego
La Jolla, California 92093-0602

REPORT DATE: June 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2002	Annual Summary (14 May 2001 - 13 May 2002)

4. TITLE AND SUBTITLE
Coactivators and Corepressors in Breast Development and Receptor-Dependent Tumorigenesis

5. FUNDING NUNUMBER
DAMD17-01-1-0185

6. AUTHOR(S)
Soo-Kyung Lee, Ph.D.
Ivan Garcia Bassets, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of California, San Diego
La Jolla, California 92093-0602

email - ibassets@ucsd.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Estrogens are important regulators of growth and differentiation in the normal mammary gland and are also important in the development and progression of breast carcinoma. Estrogens regulate gene expression via estrogen receptor (ER) and, because approximately two-thirds of all breast cancers are ER+ at the time of diagnosis, the expression of the receptor has important implications for their biology and therapy.

ER binds to the estrogen response element (ERE) found in the promoters of estrogen-regulated genes and activates their transcription. Several ER-associated proteins, coactivators and corepressors, have been identified that are of importance in regulating the ER interaction with the basal transcription machinery. Between the coactivators, a group of proteins of approximately 160 KDa molecular mass were among the first factors identified that interact with nuclear receptors in a highly ligand-dependent manner. On the other hand, in the absence of ligand, several nuclear receptors suppress basal gene transcription by recruiting corepressors.

Herein, we will address the role of the ER-associated proteins in ER action.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer, estrogen receptor, coactivators, corepressors	9
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover..... 1

SF 298..... 2

Table of Contents..... 3

Introduction..... 4

Body..... 5

Key Research Accomplishments..... 7

Reportable Outcomes.....

Conclusions..... 7

References..... 8

Appendices.....

Introduction

Estrogens are important regulators of growth and differentiation in the normal mammary gland and are also important in the development and progression of breast carcinoma (**1**). Estrogens regulate gene expression via estrogen receptor (ER) and, because approximately two-thirds of all breast cancers are ER+ at the time of diagnosis, the expression of the receptor has important implications for their biology and therapy (**2**).

ER binds to the estrogen response element (ERE) found in the promoters of estrogen-regulated genes and activates their transcription (**3**). However the details of this activation, the role of cofactors, the number and name of the gene targets, and the cross-talk between other signaling pathways are far from fully known or understood.

Recently, it has been shown that there are two different ER isoforms encoded by two different genes: ERα and ERß (**4**). Both isoforms are highly similar in the DNA-binding domain and, to a lesser degree, in the ligand-binding domain (**5**).

Several ER-associated proteins, coactivators and corepressors, have been identified that are of importance in regulating the ER interaction with the basal transcription machinery (**6**). Between the coactivators, a group of proteins of approximately 160 KDa molecular mass were among the first factors identified that interact with nuclear receptors in a highly ligand-dependent manner (**7, 8**). Expression cloning and yeast two-hybrid screening approaches led to the identification of three related genes that encode these factors, referred to as SRC-1/NCoA-1, TIF2/GRIP-1/NCoA2, and p/CIP/AIB1/ACTR/RAC3/TRAM-1 (**6**). Several lines of evidence support the idea that p160 factors play important roles as nuclear receptor coactivators, in part by recruiting CBP/p300 and arginine methyltransferase (**9**), although the extent of their role is not proven. Interestingly, p/CIP was identified and shown to be highly amplified in 10% primary breast cancers and overexpressed in 64% breast cancers examined (**10**).

On the other hand, in the absence of ligand, several nuclear receptors suppress basal gene transcription by recruiting corepressors (**6**). Nuclear receptor corepressor (NCoR) (**11, 12**) and silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) (**13**) have been intensively studied in the context of the unliganded thyroid hormone receptor (TR) and retinoic acid receptor (RAR). The amino acid sequences of NCoR and SMRT show approximately 40% identity. In contrast to other nuclear receptors, suppression of ER-mediated basal transcription by corepressors has not been observed. However, there is evidence that ER interacts with them *in vitro* and NCoR coimmunoprecipitates with ER weakly without ligand and more strongly in the presence of tamoxifen (**14**).

Moreover, it has been demonstrated that, upon the binding of the anti-estrogen TOT, ER binds to corepressors effectively. However, in the absence of N-CoR, TOT is converted from an antagonist to an agonist with regards to ER function (**14**). N-CoR expression is upregulated during late pregnancy when proliferation of mammary epithelial cells (MEC) slows and the alveoli begin to functionally differentiate by producing milk proteins (**15**). This expression profile is consistent with NCoR's function as a repressor of ER-mediated transcription since estrogen is required for proliferation of MEC, the burst of which occurs between 2-6 days of pregnancy. The ability of NCoR to funtions as a repressor of ER-mediated transactivation suggests that NCoR may play an important role not only during

normal mammary gland biology, but also in the process of breast tumorigenesis and the acquired resistance to tamoxifen treatment.

Tamoxifen is a selective estrogen receptor modulator (SERM) that competes with estrogen for binding to the estrogen receptor. In different tissues and in different animal species, tamoxifen, like other SERMs, may act as an agonist or antagonist of estrogen. In humans, tamoxifen acts as an estrogen antagonist in breast tissue, inhibiting the growth of estrogen-dependent breast tumors. In other tissues such as endometrium or bone, and in the serum lipoprotein fractions, tamoxifen acts as an estrogen agonist in that it induces endometrial proliferation, preserves bone mass in postmenopausal women, and lowers LDL cholesterol (**16**). Tamoxifen has been shown to reduce the incidence of breast cancer by more than 40% (**17, 18**).

Body

Over the initial eight months of support under the DoD Breast Cancer Research Award, I have made significant progress on several specific aims of the initial statement of work, and I have elucidated a novel intriguing regulatory event with implications for estrogen receptor resistance in breast cancer. Initially, the starting grant period was May 2001, but it was delayed to October 2001. Therefore, this report is about the period between October 2001 and June 2002.

One of the initial goals was centered about defining potential roles of p/CIP protein in the biology of mammary gland development and tumor formation. To this end, I generated a transgenic targeting vector with the p/CIP coding sequence under 7 Kbp of the whey acidic protein (WAP) promoter (details described in the Research Design and Methods of the initial proposal). The transcription unit was tested in MCF-7 breast cancer cells and proven to robustly express p/CIP transcript and protein. That construct was then microinjected into male pro-nucleus of fertilized eggs from BL6 mice, and transgenic mice where screened by Southern blot analysis for detecting the presence of the integrated vector. Three lines were selected for breeding, and all of them show to over-express p/CIP in mammary gland when tested by Northern and western blot analysis. These mice will be bred and, over the next months, will be analyzed the breast development, the response to pregnancy, lactating and involuting, and the susceptibility to tumor formation, like described in task II (initial proposal). When colony will be appropriately expanded, breeding to p/CIP and N-CoR gene deleted mice will be mutated as described under task I (initial proposal).

During the course of the initial eight months, also we have done new and very special interesting observations for further interpretation. The first related to the role of inflammation in estrogen receptor antagonist effects of target gene expression. In MCF-7 breast cancer cells, tamoxifen (4-OHT) antagonizes the activating effects of 17β-estradiol (E_2) over ERα. We have observed that this repressing effect is by recruiting N-CoR to ERα. In recent experiments, we have observed that inflammatory cytokines, such as interleukin-1 (IL-1β), can repress the antagonist effect of tamoxifen on ERα, causing fully activation of specific ER target genes. In parallel, we have evidences of the existence of a novel N-CoR complex composed by N-CoR, the histone deacetylase HDAC3, and a component of a NF-κβ

regulating complex, called TAB2. Unexpectedly, cell treatment with IL-1β causes nuclear export of the new N-CoR/TAB2/HDAC3-containing complex to the cytoplasm. Our results indicate that the molecular basis of this IL-1-dependent nuclear export is most likely to represent a MEKK1-dependent phosphorylation of TAB2 in the nucleus, putatively causing an allosteric alteration of the protein that exposes the TAB2 nuclear export signal (NES) for exporting the whole N-CoR complex to the cytoplasm. That shuttling of this complex contra poses the repressing effect of tamoxifen over ERα-target genes. To explore with more detail the specificity of this action, I propose to use a new approach that combines the immunoprecipitation of specific target DNA fragments bound to a specific protein with the microarray analysis (methodology called ChIP-chip or genome wide location analysis) (**19, 20**).

In these experiments, MCF-7 cells will be grow to 95% confluence in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% charcoal-dextran-stripped fetal bovine serum for at least 3 days (**21**). Cell cultures will be treated with IL-1β for 2 hours, and then with tamoxifen or estradiol for 1 hour. After rinsing with PBS buffer, cells will be cross linked during 20 minutes at room temperature with a PBS-1% formaldehyde solution. Cross linked cells will be harvest and kept to 30°C during 15 minutes in a Tris-DTT solution for stopping the cross linking reaction. Cellular proteins will be extracted and chromatin will be fragmented by sonication. Typically, fragments between 300 bp and 800 bp will be obtained with soft sonication. Specific proteins will be immunoprecipitated using specific antibodies: anti-ERα, anti-TAB2, anti-HDAC3 and anti-N-CoR. After reversal the cross links and DNA purification, DNA molecules will be amplified by ligation-mediated polymerase chain reaction (LM-PCR) and subsequently will be labeled with the Cy5 fluorophore by random priming (**22**). For purposes of normalization, we will perform LM-PCR on not enriched by immunoprecipitation sonicated genomic DNA and then, that amplified sample will be labeled with a second fluorescent dye, Cy3. ChIP-enriched and nonenriched pools of DNA will be mixed with Cot-1 DNA to suppress annealing of repetitive sequences and will hybridized under stringent conditions to the 1.5 Kb DNA microarray.

Dr. Bing Ren's laboratory located in the Ludwig Institute (UCSD) has developed a promoter DNA array (referred to as 1.5 Kb array) that contains polymerase chain reaction (PCR) products spanning the proximal promoters of 1444 human genes, from which approximately 1200 show regulated expression during cell cycle and approximately 200 genes doesn't show apparent link to the cell cycle (**22**). The array is composed by PCR amplified regions that span around 700 bp upstream and 200 bp downstream of putative transcription start sites. These last based on algorithms that predict DNA-binding motifs within promoters.

Those studies would open important and amazing new aspects in the understanding of ERα and cofactors action, breast cancer, and tamoxifen resistance. Basically because the use of the ChIP-chip methodology will allow to know new direct ERα-target genes and to know when the repressing complex N-CoR/TAB2/HDAC3 is recruited to repress the transcription of specific genes in response to tamoxifen and IL-1β treatments.

A parallel issue is that diverse evidence suggests a role for Wnt signaling in mammary gland development and tumorigenesis (**23**). One of them comes from LEF-1^{-}/LEF-1^{-} mice, which arrest tooth and mammary gland development at the "bud stage" (**24**). This is a stage of development when locally thickened epithelium invaginates into underlying condensed mesenchyme to form a characteristic bud structure, from which the mammary ductal system will develop (**25**). LEF-1/TCF is a family of transcriptional factors that activates the gene

expression when associated in a complex with β-catenin (**23**). This last is a coactivator protein that is shuttled to the nucleus when an external Wnt signal is recognized at the cellular membrane level by specific receptors and co-receptors, and the signal is transduced to repress the β-catenin cytoplasmic degradation pathways and Wnt signaling pathway, been ERα/estradiol pathway one of them (**26, 27**).

To know at molecular level if β-catenin/LEF-1/TCF complex or the coactivator β-catenin alone associates with ERα, I propose to purify multiprotein complexes of β-catenin in MCF-7 cells. I will construct a stable cell line expressing a fusion protein of a N-terminus mutated β-catenin and a C-terminus TAP (Tandem Affinity Purification) tag. This tag consists of a calmoduling-binding protein (CBP) and a protein A tags separated by a TEV protease cleavage site (**28**). The mutated β-catenin form is an N-terminal deleted protein that is not cytoplasmic regulated and, therefore, is directly sent to the nucleus (**29**). Nuclear extracts from the β-catenin-TAP expressing cells will be loaded in an IgG-column that specifically retains protein A tag. After extensively washes, TEV protease digestion will allow to elute β-catenin (with their associated proteins) from the column, and then the elution will be loaded in a calmodulin column in the presence of Ca^{+2}. Under these conditions, CBP is retained by calmodulin. After extensively washes, addition of EDTA will allow to elute β-catenin multiprotein complexes from the column, and using mass-spectrometry techniques, the specific eluted polypeptides will be identified.

Key research accomplishments

- Generating of a transgenic vector that express full-length p/CIP protein under the control of a mammary gland specific promoter.
- This vector is able to express the protein in transient transfection experiments using MCF7 cells.
- The construct has been microinjected to generate a transgenic mouse.
- Three selected lines over-expressed p/CIP in mammary gland have been established.

Conclusions

- The construction of a breast specific p/CIP-overexpressing transgenic line was one the main important aspects of the initial project, and its performing will allow to continue the tasks initially proposed.
- New interesting observations have been obtained in Dr. Rosenfeld lab that open new lines of study around the role of coactivators and corepressors in breast development and receptor-dependent tumorigenesis, specially in tamoxifen resistance. This allow me to pursue new experiments to perform during the next year that will be a significant progress in the understanding of the role of ER coactivators and corepressors in the initiation and progression of breast cancer as well as in susceptibility or resistance of breast cancer to tamoxifen treatment. Basically, these are the use of a new and extremely powerful

methodology, to identify new ER/cofactor gene targets and to determine the presence of ER/cofactors on them depending of the cellular conditions (tamoxifen, estradiol and Il-1β treatments).
- In parallel, recent studies show a putative relation between Wnt signaling and ER/estradiol pathways, suggesting a cross talk in breast development and cancer. The purification of the classical coactivator of the Wnt pathway, β-catenin, and the associated proteins using nuclear extracts from stable-tranfected MCF-7 cells, will help to understand the level of this relation.
- The use of the new proposed technologies and the new approaches will provide me a very useful training in the area of breast cancer biology.

References

(**1**) Russo IH and Russo J. **Role of hormones in mammary cancer initiation and progression.** *J. Mammary Gland Biol. Neoplasia.* 3(1): 49-61, (1998).
(**2**) Osborne CK. **Steroid hormone receptors in breast cancer management.** *Breast Cancer Res. Treat.*, 51: 227-238, (1998).
(**3**) Kumar V and Chambon P. **The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer.** *Cell.* 55(1): 145-56, (1988).
(**4**) Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S and Gustafsson JA. **Cloning of a novel estrogen receptor expressed in rat prostate and ovary.** *Proc. Natl. Acad. Sci. USA.* 93: 5925-5930, (1996).
(**5**) Pettersson K and Gustafsson JA. **Role of estrogen receptor beta in estrogen action.** *Annu. Rev. Physiol.* 63: 165-192, (2001).
(**6**) Glass CK and Rosenfeld MG. **The coregulator exchange in transcriptional functions of nuclear receptors.** *Genes and Dev.* 14: 121-141, (2000).
(**7**) Cavailles V, Dauvois S, Danielian PS and Parker MG. **Interaction of proteins with transcriptionally active estrogen receptors.** *Proc. Natl. Acad. Sci. USA.* 91: 10009-13, (1994).
(**8**) Halachmi S, Marden E, Martin G, MacKay H, Abbondanza C and Brown M. **Estrogen recptor-associated proteins: possible mediators of hormone-induced transcription.** *Science.* 264: 1455-1458, (1994).
(**9**) Lee YH, Koh SS, Zhang X, Cheng X and Stallcup MR. **Synergy among Nuclear Receptor Coactivators: Selective Requirement for Protein Methyltransferase and Acetyltransferase Activities.** *Mol Cell Biol.* 22(11): 3621-32, (2002).
(**10**) Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, Sauter G, Kallioniemi OP, Trent JM, Meltzer PS. **AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer.** *Science.* 277(5328):965-8, (1997).
(**11**) Horlein AJ, Naar AM, Heinzel J, Torchia B, Gloss R, Kurokawa R, Ryan A, Kamei Y, Soderstrom M, Glass CK et al. **Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor corepressor.** *Nature.* 377: 397-404, (1995).
(**12**) Kurokawa R, Soderstrom M, Horlein A, Halachmi S, Brown M, Rosenfeld MG and Glass CK. **Polarity-specific activities of retinoic acid receptors determined by a co-repressor.** *Nature.* 337: 451-454, (1995).
(**13**) Chen JD and Evans RM. **A transcriptional co-repressor that interacts with nuclear hormone receptors.** *Nature.* 377: 454-457, (1995).

(**14**) Lavinsky RM, Jepsen K, Heinzel T, Torchia J, Mullen TM, Schiff R, Del-Rio AL, Ricote M, Ngo S, Gemsch J, Hilsenbeck SG, Osborne CK, Glass CK, Rosenfeld MG, Rose DW. **Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes.** *Proc Natl Acad Sci USA.* 95:2920–2925, (1998).
(**15**) Kurebayashi J, Otsuki T, Kunisue H, Tanaka K, Yamamoto S, Sonoo H. **Expression levels of estrogen receptor-alpha, estrogen receptor-beta, coactivators, and corepressors in breast cancer.** Clin Cancer Res. 6(2):512-8, (2000).
(**16**) Pritchard KI. **Breast cancer prevention with selective estrogen receptor modulators: a perspective.** *Ann N Y Acad Sci.* 949: 89-98, (2001).
(**17**) Early Breast Cancer Trialists' Collaborative Group. **Tamoxifen for early breast cancer: an overview of the randomized trials.** *Lancet.* **351:** 1451-1467, (1998).
(**18**) Fisher B, Costantino JP, Wickerham DL *et al.* **Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study.** *J. Natl. Cancer Inst.* **90:** 1371-1388, (1998).
(**19**) Ren B, Robert F, Wyrick JJ, Aparicio O, Jennings EG, Simon I, Zeitlinger J, Schreiber J, Hannett N, Kanin E, Volkert TL, Wilson CJ, Bell SP, Young RA. **Genome-wide location and function of DNA binding proteins.** *Science.* 22;290(5500):2306-9, (2000).
(**20**) Iyer VR, Horak CE, Scafe CS, Botstein D, Snyder M, Brown PO. **Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF.** Nature. 25;409(6819):533-8, (2001).
(**21**) Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M. **Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription.** Cell. 8;103(6):843-52, (2000).
(**22**) Ren B, Cam H, Takahashi Y, Volkert T, Terragni J, Young RA, Dynlacht BD. **E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M c heckpoints.** *Genes Dev.* 15;16(2):245-56, (2002).
(**23**) Smalley MJ, Dale TC. **Wnt signaling and mammary tumorigenesis.** *J Mammary Gland Biol Neoplasia.* 6(1):37-52, (2001).
(**24**) van Genderen C, Okamura RM, Farinas I, Quo RG, Parslow TG, Bruhn L, Grosschedl R. **Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice.** Genes Dev. 15;8(22):2691-703, (1994).
(**25**) Sakakura T. **New aspects of stroma-parenchyma relations in mammary gland differentiation.** Int Rev Cytol. 125:165-202, (1991).
(**26**) Nishita M, Hashimoto MK, Ogata S, Laurent MN, Ueno N, Shibuya H, Cho KW. **Interaction between Wnt and TGF-beta signalling pathways during formation of Spemann's organizer.** *Nature.* 17;403(6771):781-5, (2000).
(**27**) El-Tanani M, Fernig DG, Barraclough R, Green C, Rudland P. **Differential modulation of transcriptional activity of estrogen receptors by direct protein-protein interactions with the T cell factor family of transcription factors.** *J Biol Chem* 9;276(45):41675-82, (2001).
(**28**) Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Seraphin B. **A generic protein purification method for protein complex characterization and proteome exploration.** *Nat Biotechnol,* 17(10):1030-2, (1999).
(**29**) Liu C, Li Y, Semenov M, Han C, Baeg GH, Tan Y, Zhang Z, Lin X, He X. **Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism.** Cell. 108(6):837-47, (2002).